ANTIBODY PRODUCTION OF THE SKIN

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by

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The present study deals with the antibody production of rabbit skin.

PART I ANTIBODY PRODUCTION OF RABBIT SKIN IN VIVO

Materials and Methods

Experimental animals : Adult albino rabbits ranging between 2.0 - 2.5 kg. in weight were used.

Antigen : crystallized egg albumin (Ea) was dissolved in physiologic saline and sterilized by Seitz filter.

Buffered saline : Buffered saline was prepared by adding one volume of a solution (0.15M) of KH_2PO_4 and Na_2HPO_4, mixed in proper proportions to produce the desired pH.

Tannic acid : Commerical product of Dai Nippon Pharmaceutical Co. was used.

Sheep erythrocytes : Sheep blood was collected aseptically in one volume of ALSVER's solution. The blood was used within three weeks. At the beginning of each experimental day an aliquot was taken and the cells washed in saline solution three times and then suspended (2.5%) either in saline or buffered saline. (pH 7.2)

All sera and tissue culture fluids were inactivated before use by heating at 56°C for 30 minutes. They were subsequently absorbed with sheep erythrocytes (one volume of packed sheep red blood cells per volume of serum) by contact for 10 minutes at room temperature.

Measurement of antibody titers :

The hemagglutination technique of BOYDEN was used. One volume of 2.5% sheep erythrocytes in 0.15 M. phosphate buffered saline (pH 7.2) was added to one volume of I : 20000 tannic acid saline solution and the mixturewas incubated for ten minutes at 37°C. The mixure was then washed once in buffered saline (pH 7.2), centrifuged at 1000 r. p. m. for 3 minutes, and resuspended in physiologic saline solution. One volume of this tannic acid treated erythrocyte suspension in saline was added to 4 volume of buffered saline (pH 6.4), containing the dissolved antigen (0.5 mg of egg albumin per ml) and left for 40 minutes at room temperature. It was then washed in 1 : 250 normal rabbit serum in saline, centrifuged at 1000 r.p.m. for 3 minutess and finally resuspended in one volume of 1 : 250 normal rabbit serum.

The serial two-fold dilution of antisera in 0.5 ml quantity was prepared with 1 : 100 normal rabbit serum in saline, and one drop of 2.5% tanned erythrocytes (0.05 ml)

was added to each test tube, shaken immediately. They were held at room temperature overnight and the results were read on the following day. To avoid the experimental errors due to technical differences and chages of temperatures, experimental materials were stored at −20°C, and the experiments were carried out at the same time on a single day.

Preparations of tissue extracts : Skin or spleen tissues were resected aseptically, measured in wet weight, then sliced into small pieces ($1 \times 1 mm^3$) and grinded with sea sand in the same or equivalent volume of physiologic saline. This suspension was extracted by freezing in aceton-dry-ice mixture and thawed at 37°C for ten times and the resulting turbid fluids were clarified by centrifugation for 30 minutes at 3000 r.p.m. As a control, the same volume of physiologic saline was injected into other sites of the abdominal wall and the skins used as control sites were resected and their extracts were titrated.

EXPERIMENT 1

ANTIBODY PRODUCTION OF THE SKIN AT THE SITE OF ANTIGEN INJECTION

Experiment A.

Two experimental rabbits received intracutaneous injections of 0.1 ml of egg albumin, each containing 2 mg, 0.4 mg, and 0.03 mg of egg albumin, into the abdominal walls.

Twenty four hours and seventy two hours after injection, skin tissue of each concentrations (three places of different concentrations on each experimental day) were resected, and antibody titers of skin tissue extracts and sera, which were bled by cardiac puncture at the time of resection, were titrated simultaneously.

Experimental results

Antibody titers of skin tissue extract at the site of antigen injection showed 1 : 10 (No. 1) and 1 : 20 (No. 2) respectively after 24 hours, and 1 : 20 (No. 1) and 1 : 40 (No. 2) after 72 hours, and no antibody was detected in the sera of the same rabbits. These results showed that the final values of antibody titers of the skin were not influenced in the presence of the excess antigen and that the antibody in the skin appeared earlier than that in the serum. But it was assumed that the amount of antibodies in the blood was too samll to be detected by BOYDEN's test, and that the antibody in the skin above-measured was the product of the local antibody formation together with the accumulation of the antibody in the circulatory blood at the site of antigen injection. Under this assumption the following experiment was pursued.

Experiment B

The experimental rabbit, which had received an injection of 2 % egg albumin (10 mg) in the form of saline solution four times every 3 days and then on the 70th day after the final injection, received 0.2 ml of different concentrations of (2 %, 0.4 % and 0.08 %) each at three different places. After 24 hours and 72 hours, the local skins at the site of antigen injection were resected and the amounts of antibodies of the skin were titrated.

This experimental result showed that the antibody titer of the skin after 24 houres was 1 : 640, and that of the control was 160, after 72 hours 1 : 1260 in the skin and 1 : 320 at the control site. On the other hand, the serum titers revealed 1 : 80, 1 : 640, 1 : 1280, on the day of the booster injection, after 24 hours, 72 hours respectively.

It was assumed that these results indicated the possibility of antibody production of the skin at the site of antigen injection and simultaneously the accumulation of the antibody in the circulatory blood at the local site.

EXPERIMENT 2

CHANGES OF THE ANTIBODY AT THE SITE OF ANTIGEN INJECTION IN THE PRIMARY RESPONSE

Experimental methods

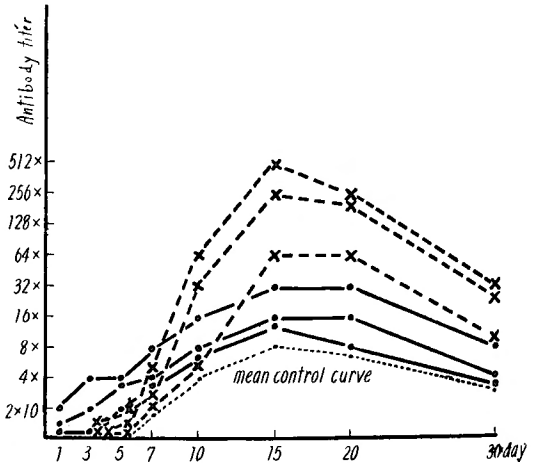
Three rabbits received intracutaneous injection of 0.1 ml of 0.5 % egg albumin solution at ten different places on the abdomen, and on the 1st, 3rd, 5th, 7th, 10th, 15th, 20th and 30th day after injection, the skins at the sites of antigen injection were resected, 500mg of these skin extracts were titrated simultaneously with the sera which were separated by cardiac puncture. On the other hand, two other rabbits which were injected with 1.0 ml of 0.5% egg albumin solution intravenously were bled by cardiac puncture on each day as above described, and the tissue extracts of the skin, as well as the sera were titrated.

Experimental results

In the group of intracutaneous immunization, the antibody titers of the skin revealed a higher value : 1 : 10 on the first day, rose gradually on the 3rd, 5th, and 7th day and reached to the peak within 10 days and then decreased gradually. Whereas the antibodies in the sera appeared on the 5th day later than that in the skin after injection, then rose rapidly to a peak much higher than in the tkin. (Fig. 1)

In the group of intravenous immunization, skin antibody appeared simultaneously with that of blood sera, both rose rapidly to the peak and later decreased gradually, but in the comparison between these two antibodies, the amounts of antibodies in the blood sera were much higher than in the skin. (Fig.2)

Fig. 1 Changes of antibody titers in the local skin of primary rabbits and in the blood by intracutaneous immunization. (Rabbit No. 4, 5, 6)
×——× Antibody titer of blood
•——• Antibody titer of skin



EXPERINENT 3

CHANGES OF THE ANTIBODY AT THE SITE OF ANTIGEN INJECTION IN THE SECONDARY RESPONSE

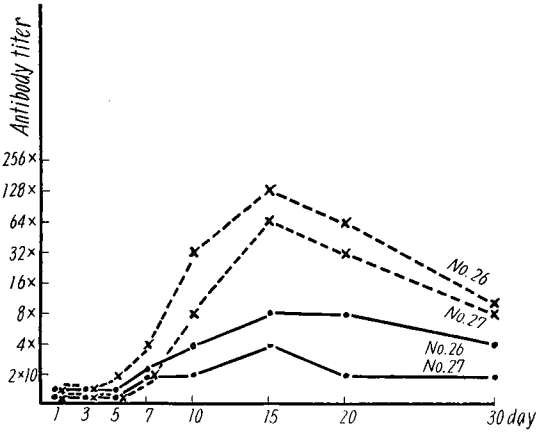
Experimenral methods

Two rabbits were injected 0.1 ml of 0.5% egg albumin solutions at ten different places into the abdominal walls intracutaneously four times every other 5 days, and at the same time and on the 1st, 3rd, 7th, 10th, 15th, 20th and 30th day after the last injections, skin tissues and blood sera were separated in the experimental use and antibody titers of these materials were titrated and compared with each other.

(the serum titer of the rabbit No. 20 revealed 1 : 5120 and No. 21, 1 : 640 on the day of final injection)

As control, the skin tissues of the sites of physiologic saline injections were resected on the 3rd, 7th, and 15th day after the final injection and the tissue extracts of these skins were titrated.

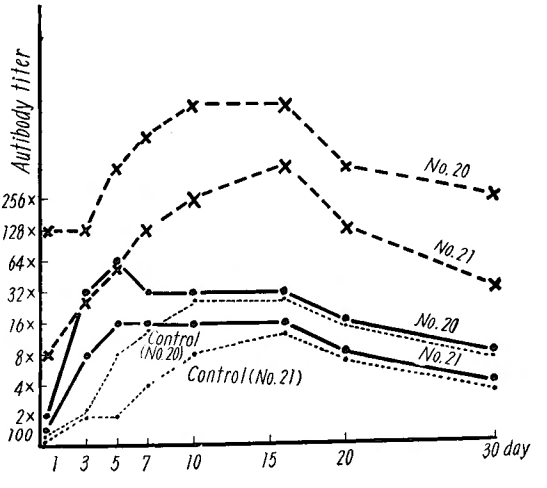
Fig. 2 Changes of antibody titers in the skins of primary rabbits by intravenous immunization. (Rabbit No. 26, 27)
×——× Antibody titer of blood
•——• Antibody titer of skin



Experimental results

The antibodies of the skin at the site of antigen injection revealed two or three fold higher antibody titers than those of the control sites and the amount of antibody in the skin was about the same as those of the serum. The concentrations of antibodies in the sera and those in the skin rose more rapidly in the secondary response than those in the primany response, reached to the peak between the 5th and 10th day and then decreaeed gradually. The serum titers were always higher than those of the skin. (Fig. 3)

Fig. 3 Changes of the antibody titers in the local skin and in the blood of secondary rabbits by intracutaneous immunization. (Rabbit No. 20, 21)
×——× Antibody titer of blood
•——• Antibody titer of skin



EXPERIMENT 4

DETERMINATION OF RESIDUAL ANTIGEN CONTENT AT THE SITE OF ANTIGEN INJECTION BY THE HEMAGGLUTINATION INHIBITION TECHNIQUE

Experimental methods

Two rabbits were injected 0.1ml of 2% egg albumin saline solution at eight places into the abdominal walls intracutaneoeously every six hours: 6, 12, 18, 24, 30, 36, 42, 48; the skin at the site of antigen injection were resected, and tissue extracts were prepared by the above mentioned method. These tissue

extracts were stored at −20°C and titrated on a single day.

The hemagglutination inhibiton test was performed by STAVITAKY's method as follows.

The serum of another previously hyperimmunized rabbit which revealed the antibody titer of more than 10000 units, was diluted with physiologic saline to contain 20 units of antibody per ml. The skin extracts were diluted with saline according to the serial two fold dilution method, then 0.5 ml of physiologic saline solution containing ten units of anti-egg albumin rabbit serum above prepared was added to each test tube. They were mixed gently and kept at 37°C for 30 minutes and finally added to these test tubes 0.05 ml of egg albumin tanned sheep erythrocytes into each tube and mixed immediately. These tubes were incubated at room temperature for 24 hours and the results read on the following day.

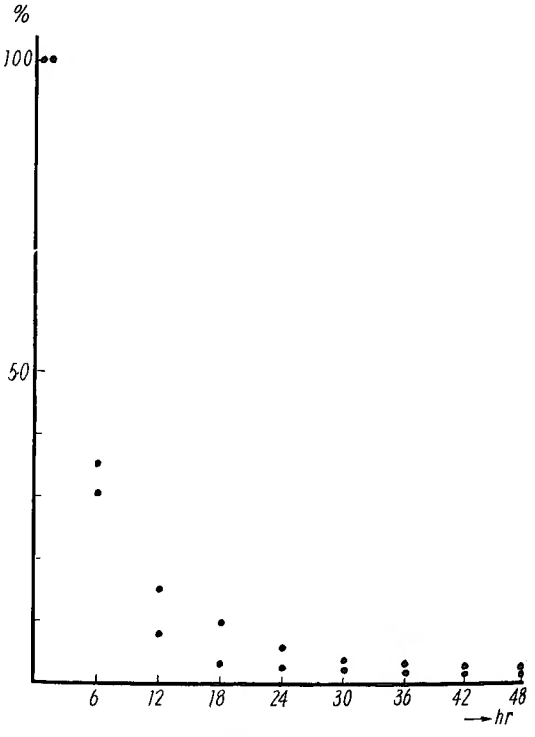
To demonstrate the antigen content quantitatively which corresponds ten units of antibody, 0.5 ml of 2 % egg albumin solution was diluted serially with ten-fold dilution series and then added to the anti-egg albumin tanned sheep erythrocytes solution (2.5%) was added. In this experiment, 0.01 mg of egg albumin corresponded to 10 units of antibody.

Experimental results

By this procedure the antigen contents at the site of antigen injection were revealed chronologically every 6 hours.

The antigen in the tissue extract combines with a definite amount of antibody; in this experiment I unit of antibody corresponded with 0.001 mg of egg albumin, and by this calculation the final point of hemagglutination inhibition reaction corresponded to 0.001mg of egg albumin. As shown in Fig. 4, the greater part of antigen at the site of antigen injection was absorbed and transported into other parts of the body within 12 hours and then it decreased gradually during the following hours. There was only less than 1 % remainder of antigen in the skin at the local site of antigen injection after 24 hours and 0.1 % of that, after 48 hours. (Fig. 4)

Fig. 4 Residual antigen content in the local site of antigen injection.



It is considered that the amount of antigen shows the antigen content in the tissue extracts and not those in the total skin because, in the residual tissue debris there remained but a small amount of antigen. However, according to the study of Korngold et al., using labeled radioactive iodine, the major part of in-

jected egg albumin disappeared from the local site of antigen injection within 24 hours, only less than 10% after 24 hours, and less than 0.1% after 48 hours remained in the local site in their experiment.

It is concluded that the antigen content in the skin at the site of antigen injection (the first amont of egg albumin was 0.2 mg) was 0.02 mg after 24 hours, 0.002 mg after 48 hours, and major part of antigen distributed to the other parts of the body through blood vessels and lymph streams.

EXPERIMENT 5

THE ANALYSIS OF THE SKIN TISSUE PROTEIN BY ZONE STARCH ELECTROPHORESIS

Experimental methods

Two albino rabbits (No. 7, No. 8) received intracutaneous injections of 0.1 ml of 2 % egg albumin (alum precipitated) repeatedly every 3 days, 5 successive times at the same site of the abdominal wall. After the final injections, on the 5th day, the skin of the injected site was resected and 1 g of the skin tissue extracts were prepared.

The apparatus of zone starch electrophoresis in the laboratory of the Microbiology at Kyoto University Medical School was used. An amount of 0.5 ml of these extracts were packed into a glass tube (30 cm in length, 2 cm in diameter) with the starch which had been dissolved in phosphate buffered saline (pH 8.0, 15 M). This apparatus was operated at 10 mA, 150 volts for 7 hours, and then the zone of the starch was cuti nto segments 5 mm in width, dissolved with 1.5 ml of physiologic saline, the amount of eluted protein into the saline was measured by the FOLIN's phenol reagent at the wave length of 610 millimicron using photoelectric equipment and 0.5 ml of another portion of this saline solution containing eluted protein was titrated in the amount of antibody by BOYDEN'S method.

Another rabbit was sensitized with 1 ml of 2 % egg albumin on the first day and 1 ml of this solution on the 7th day by intravenous immunization. On the 15th day after the first injection, then the rabbit was bled by cardiac puncture and its blood serum was separated, inactivated at 56°C for 30 minutes and simultaneously on the same day, 1 g of the skin tissue extract was analysed by zone starch electrophoresis and then measured in the protein constitution simultaneously with the antibody content by BOYDEN'S method.

Experimental results

The electrophoretic patterns of the skins of both intracutaneously immunized rabbits and intravenously immunized rabbit revealed the normal patterns of anti-egg albumin rabbit sera, distributing albumin, alpha, beta, gamma 1, and gamma 2 fractions. But in the distribution of anthbodies by BOYDEN'S method, the antibodies of the skin tissue extracts of the intrancutaneously immunized rabbits were identified in the fractions of gamma globulin, simultaneously in the fraction of alpha, beta globulin, consisting of two peaks of antibody concentration curve. On the other hand, the skin tissue antibody of intravenosly immunized rabbit was identified in the fraction of gamma globulin and with the same result as those in the early stage of immunization. (Fig. 5, 6, 7)

Fig. 5 Electrophoretic pattern of the rabbit skin of intracutaneous immunization by zone starch electrophoresis and distribution of antibody in the same starch segments. (Rabbit No. 7)
×——× Antibody titer by Boydens method
•——• Protein content by Folins phenol reagent method (μg N)

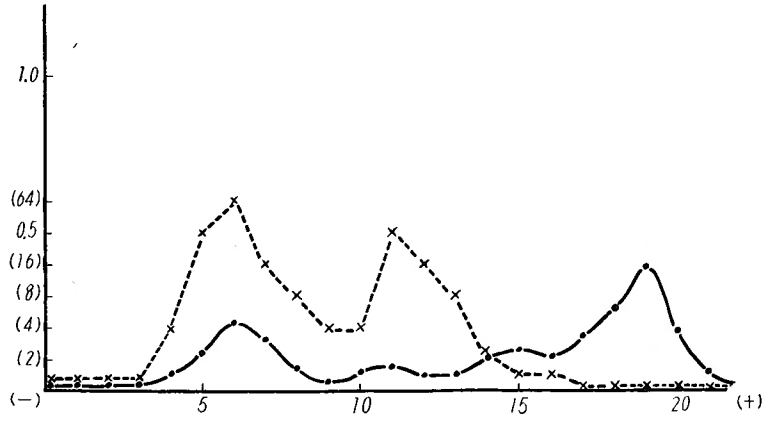
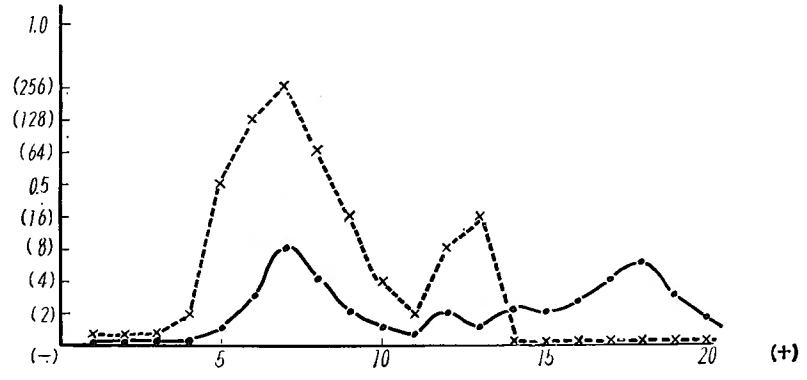


Fig. 6 Electrophoretic pattern of the rabbit skin of intracutaneous immunzation by zone starch electrophoresis and distribution of antibody in the same starch segments. (Rabbit No. 8)
×——× Antibody titer by Boydens method.
•——• Protein content by Folins phenol reagent method. (μg N)



EXPERIMENT 6

ANTIBODY PRODUCTION OF THE SKIN BY THE TRANSFER METHOD

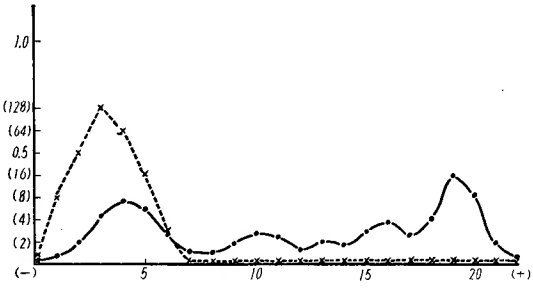
EXPERIMENT A

One donor rabbit received intracutaneous injections of 2% egg albumin saline solution into four quadrants at the abdominal walls (each quadrant received 2.0 ml of 2% egg albumin). On the 3rd and 5th day after the injection, each quadrant of the local skin was resected and sliced into pieces smaller than 1 mm^3 and then 1 g of these tissuss were

transfered aseptically into the subcutaneous space of one recipient rabbit to the abdominal wall on each transfer day. On the lst, 3rd, 5th and 10th day after transfer, blood sera were collected by cardiac puncture and the antibody titers ofthe recipient rabbits to egg albumin were titrated by BOYDEN's method.

As control, the sliced skin of the donor rabbits was inactivated by freezing and thawing for ten times rapidly and then transfered into the subcutaneous spaces of the recipient rabbits, and the serum of the recipient rabbits were titrated chronologically on the same experimental day according to the above mentioned experimental schedules.

Fig. 7 Electrophoretic pattern of the rabbit skin of intravenous-immunization by zone starch electrophoresis and distribution of antibody in the same starch segments. (Rabbit No. 9)
×——× Antibody titer by Boydens method.
•——• Protein content by Folins phenol reagent method. (μg N)



Experimental result

No antibody was revealed in the recipient rabbits as well as in the control rabbits.

EYPERIMENT B

One rabbit received intracutaneous injections of 2% egg albumin into four quadrants at the abdominal walls two times at the interval of ten day (each quadrant received 2.0 ml of 2% egg albumin solution on the day of injection).

On the 3rd, 5th, 7th and 15th day after the final injection, each quadrant of the local skin at the site of antigen injection were resected and sliced into pieces and then 1 g of these tissues were transfered aseptically into two other recipient to the abdominal wall on each transfer day, as follows; one rabbit received the skin tissue slices of the donor rabbit intraperitoneally, another rabbit received intracutaneously. On the 1st, 3rd, 5th, 10th and 15th day after transfer, blood sera were bled by cardiac puncture and the antibody titers of the recipient rabbits to egg albumin were titrated by BOYDEN's method.

EXPERIMENT C

To prove the site of antibody production of the skin more precisely, the epidermal layers of the two donor rabbits were separated from the dermal layers of the resected skin with razors and each layer (4) was kept separately; the epidermal layers of the two rabbits were collected; the transfer of the epidermal layer shows the transfer of the sum of the two donor rabbit; and simultaneously the dermal layer of each one rabbit were transfered on the each transfer day: One gram of each portion of donor rabbits, which received injections of antigen, in the same amount and according to the immunization schedule as experiment B, was transfered into recipient rabbits, on the 1st, 3rd, 5th, 7th and 15th day after the 2nd injection according to the above dercribed procedure. The sera of the recipient rabbits were titrated on the 1st, 3rd, 5th, 7th and 15th day after transfer. All sera were stored at −20°C and inactivated at 56°C ofr 30 minutes before titration.

Experimental results

The amount of antibody formed in the recipients was determined as follows: Total antibody formed in the recipient was calculated by subtracting from the antibody titer, the amount of antibody which could have been present merely as contamination of the serum with antibody, associated with the transplanted tissue. This basal amount of antibody was derived by measuring the antibody content of an extracted aliquot of the tissue fragment, multiplying this figures by a number depending upon the volume of cell suspension injected, and then dividing it by the volume of the recipient. According to the latter figure the plasma volume represents 3.21% of the body weight. (A. B. STAVITSKY)

On the 3rd day after the transfer, antibody titer to egg albumin reached the peak in some recipient rabbit and other rabbit within 7 days. In some recipient rabbit the antibody titer reached the maximum value of 1 : 1024. (Fig. 16)

The transfer of the dermal layer yielded large amount of antibody in the recipient, whereas the transfer of the epidermal layer yielded little or no antibody in the recipient. (Fig. 8～Fig. 15)

The control rabbits into which were transfered, inactivated, inactivated non viable skin tissue of the donor rabbits revealed no antibady production within 15 days after transfer.

The transfer on the 15th day after the antigen injection revealed comparatively high amounts of antibodies in the recipient. (Fig. 14)

In these transfer experiment, two objections may be presented: the possibility of active immunization by antigen with the transfered tissues and the possibility of passive immunization by

Fig. 8 Antibody titers of the recipient rabbits. (rabbit No. 32, 33)
Donor skins were transfered into recipient rabbits on the 3rd day after antigen injection.
•——• Subcutaneous transfer
×——× Intraperitoneal transfer

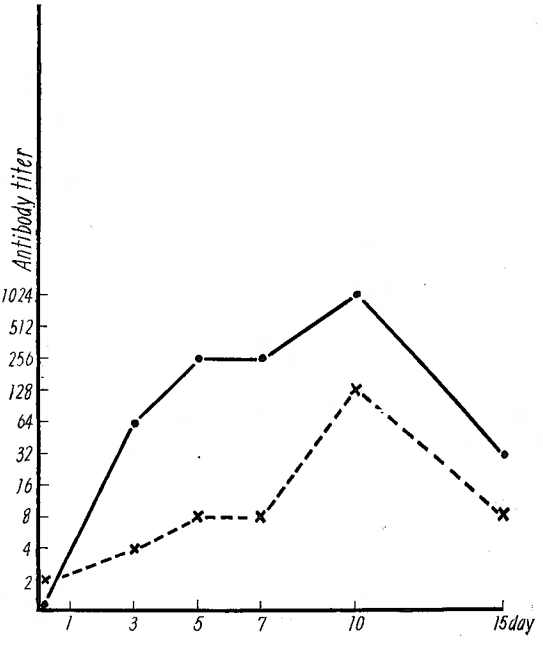
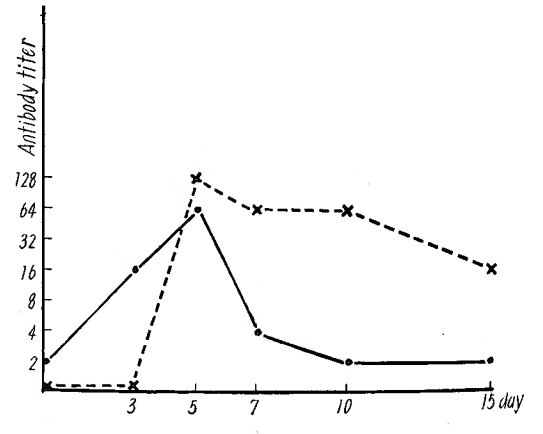


Fig. 9 Antibody titers of the recipient rabbits. (Rabbit No. 34, 35)
Donor skins were transfered into recipient rabbits on the 5th day after antigen injection.
•——• Subcutaneous transfer
×——× Intraperitoneal transfer



the antibody with the transfered tissues. The passively transfered antibody in the recipient were assumed to be the amount of 1 : 40 or 1 : 80 per tissue (by BOYDEN'S method) and this amount of antibody may be diluted in the recipient plasma, showing little or no antibody titer in the serum of the recipient rabbit. Whereas the amount of antibody produced in the recipient rabbits were too great, reaching the maximum value of 1 : 64 and 1 : 1024; in the total amount of 10000 or 120000 units. This value is not consistent with the amount of passively transfered antibody. The curve of passively immunized rabbit showing the rapid increase and rapid decrease within one or two days, differs from that described in this experiment. The antigen introduced into the recipient with the transfered tissue may be less than 50 micrograms by the calculation of Experiment 3, and this small amount of antigen should not produce an appreciable amount of antibody in the recipient by BOYDEN'S method. In the further experiment, some recipient was injected 1 ml of 2 % egg albumin solution 4 weeks after transfer and the recipient produced small amount of antibody in the primary response and none in the secondary response. Under these consideration, the possibility of both active and passive immunization of the recipient was excluded and it was proved that the viable cells in the skin of the donor rabbit did respond with the production of antibody in the recipient.

Fig. 10 Antibody titers of the recipient rabbits. (Rabbit No. 36, 37)
Donor skins were transfered into the recipient rabbits on the 7th day after antigen injection.
• —— • Subcutaneous transfer
× —— × Intraperitoneal transfer

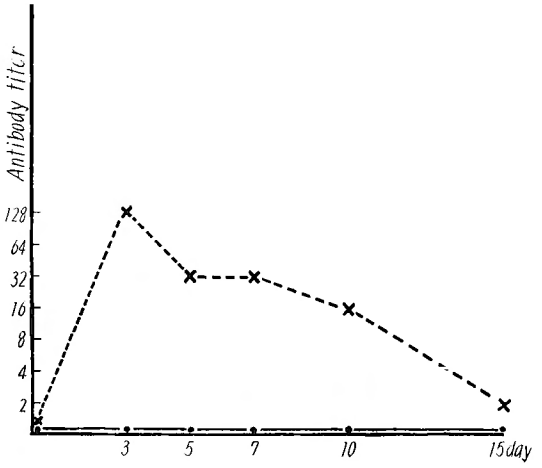
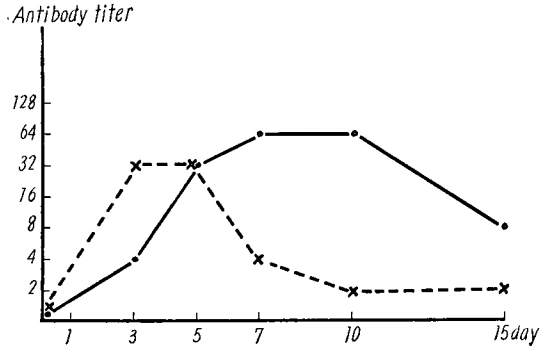


Fig. 11 Antibody titers of the recipient rabbits. (Rabbit No. 43, 44)
Donor dermis were transfered into recipient rabbits on the 3rd day after antigen injection.
• —— • No. 43 × —— × No. 44



In addition to this results, it was found that the major part of antibody production in the skin existed in the dermal layer and not in the epidermal layer.

PART 2

ANTIBODY PRODUCTION OF RABBIT SKIN AND SPLEEN IN VITRO

Materials and Methods

Experimental animals: adult albino rabbits weighing 2.0～2.5 kg were used.

Antigen : Crystalline egg albumin

Serum : Rabbit autoserum was separated before experiment, inactivated at 56°C for 30 minutes, and stored at 4°C or −20°C in the refrigerator.

Culture medium: 10× Hanks solution was stored at 4°C in the refrigerator. At time of use, this solution was diluted with twice-distilled water, heated by autoclave at 10 pounds for 10 minutes. An amount of 1.4% $NaHCO_3$ was autoclaved at 10 pounds for 10 minutes, and this solution was added to 20 cc of Hanks solution with 0.5 cc.

Lactoalbumin hydrolysate of Difco was added in the concentration of 0.5 % into the Hanks solution according to the procedure of Melnick.

Culture Method : The spleen or the skin tissues of the antigen injection and the normal tissue were resected aseptically (in the case of skin resection, the hairs on the skin were cut and rinsed with sterile physiologic saline, sterilized with 70 % alcohol, and finally resected aseptically).

The tissues were washed three times with sterile Hanks solution, adjusted to a pH of 7.4 with sterile $NaHCO_3$, and then sliced into pieces smaller than $1mm^3$, 500 mg of these sliced tissues were cultured in the culture media of MH medium 8 cc (Hanks solution at pH 7.4 containing 0.5 % lactoalbumin hydrolysate) and rabbit serum 2 cc, in the prescription bottle of 50 cc in volume at 37 °C.

The culture media were replaced with the fresh culture media of 0.5 cc containing the same constituents of the culture media above described, on the 1st, 2nd, 3rd and 4th day after incubation antibody titers of the culture media were titrated.

Fig. 12 Antibody titers of the recipient rabbits. (Rabbit No. 45, 46)
Donor dermis were transfered into recipient rabbits on the 5th day after antigen injection.
•——• No. 45 ×——× No. 46

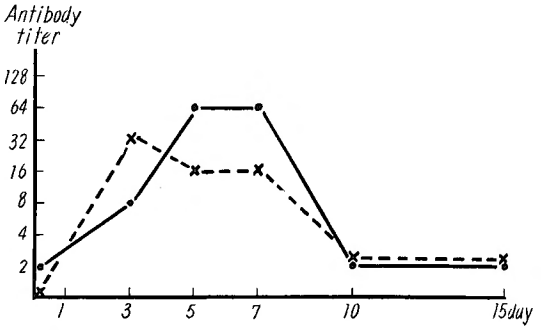


Fig. 13 Antibody titers of the recipient rabbits. (Rabbit No. 47, 48)
Donor dermis were transfered into recipient rabbits on the 7th day after antigen injection.
•——• No. 47 ×——× No. 48

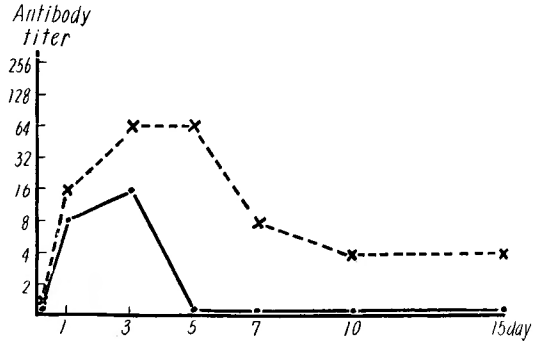
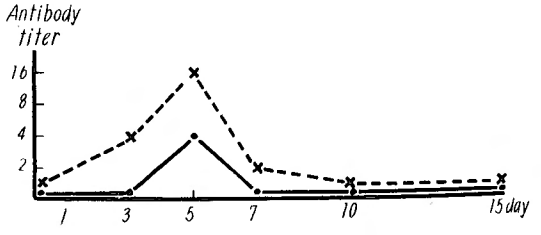


Fig. 14 Antibody titers of the recipient rabbits. (Rabbit No. 49, 50)
Donor dermis were transfered into recipient rabbits on the 15th day after antigen injection.
•——• No. 49 ×——× No. 50



EXPERIMENT 7

ANTIBODY PRODUCTION OF THE SKIN AND THE SPLEEN IN VITRO BY THE INITIATION WITH THE INTRODUCTION OF ANTIGEN TO THE TISSUES IN VITRO

Experimental methods

Two rabbits were sacrificed by total bleeding and the skin at the abdominal wall were resected aseptically and each 500 mg of the spleen and skin were

Fig. 15 Antibody titers of the recipient rabbits. (Rabbit No. 51, 52, 53)
Donor epidermal layers were transfered into recipient rabbits on the 3rd, 5th and 7th day after antigen injection.
•——• Transfer on the 3rd day.
×——× Transfer on the 5th day.
△------△ Transfer on the 7th day.

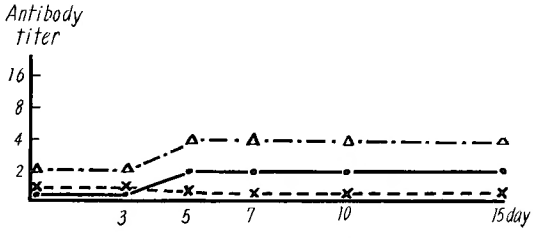


Fig. 16 TRANSFER EXPEPIMENT
Schedule of immunization and time of removal of skin

Donor rabbit	Mode of immunization	Length of interval between booster injection and time of removal	Serum titer
No. 31	※ IC·····IC	3 days	160
		5 days	160
		7 days	320
No. 41	IC·····IC	3 days	120
		5 days	120
		7 days	240
		15 days	480
No. 42	IC·····IC	3 days	160
		5 days	160
		7 days	320
		15 days	800

RECIPIENT RABBITS

Lengfh of interval between booster injection and time of removal		Reciprocal peak titer	Days after transfer
No. 31	3 days	1024	10
	5 days	128	5
	7 days	128	3
No. 41	3 days	64	3
	5 days	64	5
	7 days	64	3
	15 days	16	5
No. 42	3 days	32	7
	5 days	32	3
	7 days	16	3
	15 days	4	5

※ I. C; Intracutaneons

sliced into pieces, incubated in the Hanks solution at 37°C for one hour conta ing 0.5 % egg albumin, and after this washed with the fresh Hanks solution several times, finally these tissuee were cultured. On the 1st, 2nd, 3rd and 4th day after incubation, the antibody in the culture media were titrated by BOYDEN'S method.

Experimental results

Both spleens and skins of two rabbits yielded not any antibody to egg albumin in the culture media.

EXPERIMENT 8

THE ANTIBODY PRODUCTION OF THE SKIN AND THE SPLEEN IN VITRO OF THE PRIMARY RABBIT IN THE EARLY STAGE OF IMMUNIZATION

Experimental methods

One rabbit received intracutaneous injections in the abdominal wall (0.1 ml of 2 % egg albumin in each place, total 5 places) other two rabbits received intravenous injections of 2% egg albumin through the marginal ear vein, and these two rabbits of intravenous injection were sacrificed by total bleeding on the 1st and 3rd day after injection, their spleens and the skin at the site of antigen injection of intracutaneously immunized rabbit were resected, cultured by the same culture method above described. On the 1st, 2nd, 3rd and 4th day after incubation, the culture media were titrated in the amount of antibody content.

Experimental results

The skin tissues of both intracutaneous immunization and intravenous immunization yielded no antibody titer in the culture media, on the other hand the spleen of intravenously immunized rabbit resected on the first day after injection revealed the antibody titer of 20 units and the spleen of intravenously immunized rabbit resected on the 3rd day after injection revealed 80 units of antibody in the culture media.

EXPERIMENT 9

THE ANTIBODY PRODUCTION OF THE SKIN AND THE SPLEEN IN VITRO AFTER THE INTRAVENOUS INJECTION OF SALMONELLA TYPHOSA VACCINE AS A PROVOCATION

Experimental method

Two cc of the heat-killed typhosa vaccine at 60°C for 30 minutes (2 mg of bacteria in wet weight) was injected into the marginal ear vein, and two days later 0.5 cc of 1 % egg albumin saline solution was injected intravenously through the ear vein, at the same time 0.1 ml of this solution was injected intracutaneously into the abdominal wall of 5 places.

Twenty four hours after injection, the rabbit was sacrificed by total bleeding, the skin of the antigen injection and the spleen were resected aseptically, and 500 mg of each tissue in wet weight were cultured in 10 cc of MH 80, rabbit autoserum 20. On the

1st, 2nd, 3rd and 4th day after incubation the antibody titers of the culture media were titrated.

Experimental results

The skin tissue yielded no antibody in the culture media but the culture media of the spleen revealed 1 : 40 in antibody titer by BOYDEN'S method on the 1st, 2nd, 3rd and 4th day after incubation.

EXPERIMENT 10

ANTIBODY PRODUCTION OF THE SKIN IN THE SECONDARY RESPONSE IN VITRO

Experimental methods

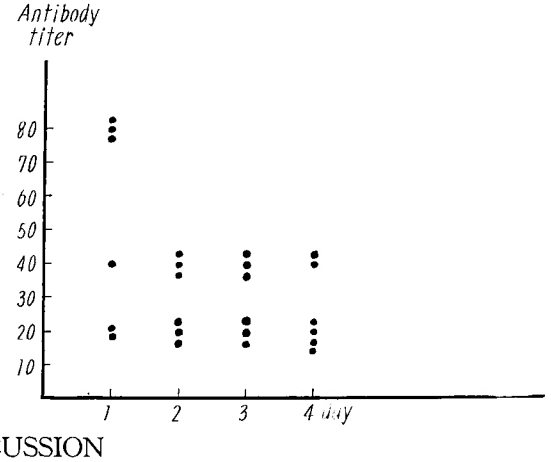
Six rabbits (No. 30, No. 35, No. 36, No. 37, No. 38, No. 40) received intracutaneous injections of 1 % egg albumin solution; 0.1 ml of antigen solutions into each of 5 places in the abdominal walls; 3 months later booster injections were done as to the same schedule, and on the 3rd day the skin tissues of the abdominal walls were resected, 500 mg of each skin tissue were sliced and cultured by the same method above described, and the antibody titers of the skin eluted into the culture media were titrated by BOYDEN'S method.

Experimental results

The culture media of the skin revealed the antibody titers of 1 : 10 and 1 : 20 and these antibodies were specifically inhibited by the egg albumin, using the hemagglutination inhibition test. The total amount of antibodies produced in the culture media should be 200 or 400 units in BOYDEN'S hemagglutination unit, whereas the antibody content of the cultured skin at the time of resections may be calculated 40 units in BOYDEN'S unit. (Fig. 17)

In these results, the antibodies detected in the culture media of the skin were actually produced in vitro.

Fig. 17 Antibody content of the culture media of the secondary rabbit skins.



DISCUSSION

The existence of antibodies in the skins of the immunized rabbits is proved by the evidences of their existence in tissue extracts of the skins, but whether these antibodies in the skin were produced by the skin itself or by other organs of the body and then transported to the skin, it is not certain. There is but a small amout of antibodies in the skin as revealed by the micromethod, and the antibodies in the skin consist of antibodies of the skin itself plus antibodies in the circulatory blood produced by other organs. It is difficult to discriminate these antibodies quantitatively and qualitatively.

FREUND et al. found that in the study of passively immunized rabbits the antibodies

in the skin at the site of injections were higher than those at the control sites for the first five days and disappeared from the skin from 6 to 12 days. Six days later, the antibodies distributed in the blood and organs as if they had been injected directly into the blood stream. In another of his experiments, using typhoid immune sera, the skin was perfusion-fast with physiologic saline in compared with other organs such as spleen and liver. However, the antibodies in the skin were difficult to draw out by rinsing with physiologic saline, and furthermore the distribution of antibodies in the serum and organs of actively immunized rabbits were similar to those in passively immunizedr abbits 7 days later. The ratio between the titer of the serum and the organ extracts such as skin and spleen were 10 : 0.6 or 10 : 0.25.

OAKLEY et al. studied the antibody production of the skin in view of the local immunity, and the ratio of the antibody content of the tissue extract of the skin to the antibody content of the serum increased gradually during 30 days after immunization, reached the peak at the value of 4.0, and later this value persisted for one or two months. These facts suggest that the antibody production of the skin increases more slowly than that of the serum, but higher levels of the antibody of the skin persist for comparatively longer periods than those of the serum. In the Experiment 1, 2, and 3 antibody production of skin in its early stage of immunization was studied ; it was revealed the the presence of antibodies in the skin with the absence of serum antibody in that stage.

The appearence of antibody in the skin in the early stage of absence of serum antibody suggests that the antibody production is present in the local skin of antigen injection and this antigen combine with the circulatory antibody in the blood, and this antibody concentrate at the site of antigen injection, and then this accumulated antibody at the site of antigen injection is proved by BOYDEN'S test. These facts were also proved by Coons with the fluorescin labelled antibody technique. In Experiment 1, 2, the skin tissue at the site of antigen injection revelaed two or three fold higher antibody contents than those at the control site in the secondary response. It is impossible to divide the antibodies of the skin into two parts ; the antibody produbed in the skin itself, and the antibody in the circulatory blood, in Experiment 1, 2, 3. Many studies have been made in recent years on the various qualities of antibody, but no definite conclusion has not yet been reached. KAGEYAMA reported in the study of the spleen in vitro that, in the culture media of spleen resected in the early stage of intravenous immunization, antibodies were identified in the fraction of albumin by hemagglutination inhibition test and albumin test, in the fraction of beta globulin by antiglobulin test and albumin test, and other antibodies were revealed in the fraction of gamma globulin by antiglobulin test, BOYDEN'S test, and precipitin test. In the studies of many investigators, incomplete antibodies were mainly proved in the fraction of non gamma globulin, and complete antibodies were proved in the fraction of gamma globulin. Moreover, it was proved by KAGEYAMA's studies that the antibodies eluted into the culture media of spleen and lymphnodes in the early stage of immunization, appeared first in the fraction of beta-globulin and later within several days, shifted to the fraction of gamma globulin. It was assumed by these experiment that these antibodies in the fraction of beta globulin were the precursors of the antibody in the fraction of gamma globulin. In addition to this fact, after several days,

in the culture media of spleen and lymphnodes and also in the sera from intravenously immunized rabbits, one peak of antibody distribution was found in the fraction of gamma globulin.

In the studies of zone starch electrophoresis, presented here, antibodies in the skin extracts of the antigen injection of intracutaneously immunized rabbits, distributed in the fraction of beta gamma globulin, exhibiting two peaks. On the other hand, antibodies of the skin exracts of intravenously immunized rabbits distributed only in the fraction of gamma globulin in the form of one peak. Those results certified the existence of different qualities of antibodies in the skin at the site of antigen injection and support the theory of the antibody production of the skin. In the transfer experiment, TOPLEY found that the increase otf heantibody in the blood of the recipient rabbits was proved when the spleen of the donor rabbits were transplanted into the recipient, and this antibody production of the recipient rabbits reached the peak in the comparatively shorter incubation periods, whereas the passively transfered immune sera in the recipients reached the maximum within 24 hours, and then decreased gradually; and in the case of antigen injection, the incubation periods were comparatively longer. And when these recipient rabbits received the same antigen repeatedly, these rabbits proved positive in the primary response and negative in the secondary response. He assumed by these results that the antibodies of the recipient were not the results of the transfered antibodies with the trasfered cells but the antibodies of the antibody produding cells.

HARRIS and HARRIS found that it was necessary to use viable cells for the production of antibody in the recipients by the transfer method, with the results of no antibody production by the transfer of the lymphnode suspension which had been inactivated by freezing and thawing or incubation at 37°C for 24 hours or incubation at 52°C for 20 minutes. It was also proved by his experiments that the antibody production of the recipients which had been irradiated by X ray and had been reduced in the antibody producing capacity previously were not reduced, rather induced higher in the antibody production. By these results they presumed that the transfered cells have the successive antibody producing capacities in the recipients. DIXON and WEIGLE found the necessity of using adult recipient animals to produce the antibody because that can not be done in the new born rabbits. These results show the importance of the environment of the transfered cells. STAVITAKY studied the various factors of the transfer experiments and found that the main factors of antibody production in a case of intravenously immunized rabbit were the spleen and bone marrow, and regional lymphnode. The main part of the antibody production of the transfer was the regional lymphnodes in the case of antigen injection into the foot hind pad of the rabbit, and the transfer of the opposite part of the regional lymphnodes of the food pad revealed no antibody production in the recipients; and moreover the transfer of the interscapular granuloma produced by the injection of alum precipitated egg albumin proved antibody production in the recipients. As to the factors related to the recipients, the antibody production of transfer method were only successful by the homotransplantation. For instance, it was not possible to produce the antibody in the transfer of the tissue from rabbit to rat, but in the transfer of rabbit. According to these above described experiments, the antibody production by the transfer

method was not initiated by the active immunization or by the passive immunization. It may be assumed that the antibody production of the recipient by the transfer method was related to the transplanted tissue and other factors such as recipient and donor factors and transfer method. The present data and other studies by other investigators strongly suggest the evidence for the autonomy of the donor cells in antibody production, and correspond to the transfer experiment of the skin.

Since the application of the tissue cultures to the antibody production of the organs or tissue cells in vitro by CARREL and INGEBRIGSTEN in 1913, many investigations have been made in this field. In Japan, Professor R. Kimura and his co-workers have made many valuable investigations. The studies of antibody production in vitro may be divided into three major methods; the first is the antibody production of the tissue of the animals (hyperimmune) ; the second is the antibody production of the tissue of the animals in the early stage of immunisation; the third is the antibody production of the tissue of the non-immunized animals with the initiation of immunization by in vitro contact method.

Antigenes which were introduced into the experimental animals, were assumed to be changed into the intermediate form or precursors of antibody, and then transfered to antibody producing cells or organs, and in these organs or cells capable of producing antibodies. In the immunization of the experimental animals, it is proved that the primary response appears within 2 or 7 days after the latent or negative periods. During these intervals, antibody producing cells increase in number by mitosis and replicaton, and the antigen introduced into the cells was transformed into the intermediate form of antibody, and then after about 7 days of induction period, antibody production in the experimental animals markedly increase. The first procedure of antibody production in vitro of the hyperimmune rabbits is started with the initiation of the immunization in vivo and the production of antibody performed in vitro. The 2nd procedure of the antibody production in vitro was that the cultured tissues were resected in the early stage of immunization within 7 days and then the antibody production in vitro was measured. The third method of the antibody production was initiated in vitro with the contact of antigen, and then these initiated tissues were cultured in vitro and measured in the antibody production.

Until now the major organs or tissues were the spleen or lymphnodes such as reticuloendothelial system, and the studies of antibody production of the skin in vitro had been very rare. MURAKAMI reported the antibody production of the skins which were resected after the treatment of antigen with rabbit in the form of Lanolin ointment, and found in the culture media of the skin small amounts of agglutinin to the typhoid bacillus. STAVITSKY reported the production of small amounts of antibody in the culture media of the alum granuloma formed in the subcutaneous site of rabbits, whereas the spleen and the regional lymphnodes of the same rabbit produced considerable amounts of antibody.

In our studies presented here, antibody production of the skin was proved in the early stage of secondary response, but not proved in the primary response rabbit. The reason for no antibody production of the skin in vitro in the primary response was assumed to be the fact that the cells in the culture media used in this experiments were not under optimum conditions, because that the antibody production of the skin in vitro requires more adequate environment such as pH, culture temperature, especially the constitution

of the synthetic culture media.

In Experiment 8, the primary rabbit which had been provoked by the typhoid vaccine injection before the resection of tissue failed to produce the antibody in the culture media of the skin, according to the method of McKENNA and STEVENS.

In the transfer experiment, the transfered tissues of the secondary rabbits were capable of producing antibody in the recipient while those of the primarily stimulated rabbits were not capable. These results of the transfer experiments correspond with the results of the experiments by the tissue culture method.

There are many hypothesis and evidences to elucidate the marked differences of antibody production between the primary response and the secondary response. GLENNY considered that antibody production may be performed in two steps: the sensitization phase and the production phase. In the first step, antibody producing cells were effected with the contact of antigen, and then in the second step, antibody may be produced with the second contact of antigen. It was assumed by JERNE, that antibody production may be possible in the presence of antigen-antibody complexes in the animal. Antigens which had been introduced into the body through the intracutaneous route disappeared at the site of injection, i. e., 24 hours after injection, approximately one half of the diphtheria toxoid was destroyed, and in the second step, the remaining antigen deposited in the alum granuloma was eliminated slowly as the results of a partial break-down at the site of inoculation, and the passage of a small constant proportion of intact toxoid to the draining lymphnode was proved. In this paper presented here, two thirds of the soluble antige nwas catabolized at the site of antigen injection. The antigens which were intrcduced in animals distributed mainly into the macrophage system. But recently the antibody produced in cells was identified in the microphage system, by the study of the fluorescin labeled antibody technique. The major part of spleen tissues consists of the microphage system and that of the skin consist of the macrophage system. The antibody production of the spleen in vitro was higher than that of the skin in vitro in this study. In the tissue culture of the rabbit skin, the epithelial cells migrate within 24 hours earlier than the cells of the macrophage systhem such as histiocytes and lymphocytes and plasma cells (with the cells of microphage system). These two systems are related to the antibody production.

The macrophage system stores the injected antigen, transforms the particulate antigen into a soluble form in the cells, and then this altered antigen is transfered to the antibody producing cells and in this places antibody production may be produced.

In other words, the macrophage system plays the major part of antibody production in the induction phase, and also the microphage system plays the major part of the antibody production in the production phase. In the analysis of the skin at the site of antigen injection by the zone starch electrophoresis, the antibody in the fraction of alpha and beta globulin may be the precursor of the complete antibody. Therefore, it is evident that the antibody production may be actually present in the skin at the site of antigen injection.

It is not certain in what mechanism the cells in the skin participate in antibody production. The hypothesis that the antigen does not immediately gain access to the active site can be eliminated on the basis of the successful transfer of antibody synthesiz-

ing cells within a few minutes after the injection of antigen into the donor rabbits. In the latest experiment of McKENNA and STEVENS, macrophage cells in the peritoneal cavity of rabbit produced antibody directly with the addition of antigen in tissue culture. Coons et al., using the fluorescin labeled antibody techniqe, detected the gamma globulin in the histiocytes and collagen fibers in the granuloma formed by the alum precipitated antigen at the local site of the antigen injection.

With these results and considerations above described, the macrophage cells cooperate in the antibody production of the skin.

CONCLUSIONS

In the present experiment, antibody production of rabbit skin using the hemagglutination by BOYDEN'S method were studied and the results are as follows:

1) When the soluble antigen of the crystalline egg albumin was injected into the abdominal wall intracutaneously, tissue antibody of the skin appeared earlier than those of the serum, within 3 days, and later the amount of antibody in the skin was paralelled with the increase in the serum. In the course of antibody response, both of the primary and secondary, the amount of autibody of the serum was much higher than those in the skin.

2) Antigens which was introduced into rabbits decreased at the site of antigen injection; two thirds of the introduced antigens were transported into another site of the body within 24 hours and then gradually decreased afterwards.

3) Antibody concentration at the site of inoculation was two or three fold higher than those of the skin at the control site.

4) The antibodies of the skin at the site of inoculation with 2% alum precipitated egg albumin distributed in the fraction of alpha, beta, and ga mma globulin which were isolated by the zone starch electrophoresis, whereas the antibodies of the skin of the rabbits after intravenous immunization of the same antigens distributed in the fraction of gamma globulin. It is conceivable that these antibodies identifid in the fraction of alpha and beta globulin are the precursors of the antibody in the incomplete form of antibody and this result suggests the antibody production of the skin from the view point of qualification of antibody between the locally produced antibody of the skin and the circulatory antibody in the skin.

5) The skin of the donor rabbits at the site of antigen injection in the early stage of immunization were transfered into the recipient rabbits, and antibody production of the recipient rabbits was proved in the serum within 7 days, reached the peak and then decreased rapidly.

In the additional experiments by the transfer method, the main portion of antibody production of the skin existed in the dermal layer of the skin and not in the epidermal layer.

6) Antibody production of the skin at the site of antigen injection of the secondary rabbit was proved in tissue culture but failed to produce antibody in the culture of primary rabbit skin. It may be possible to produce more antibody under more optimum culture conditions in the tissue culture of the skin of the primary and the secondary response rabbits.

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和文抄録

皮膚の抗体産生について

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家兎を用いて，皮膚の抗体産生能を Boyden 法を用いて追及し，次の結果を得た．

1) primary response の家兎の卵白アルブミン抗原注射局所には流血中抗体の出現に先立つて皮膚組織抗体を立証した．

2) Secondary response の家兎皮膚では抗原注射局所の組織抗体は対照の生理食塩水注射局所の皮膚組織抗体より2～3倍程度高い抗体を保有していることを立証した．

3) 抗原注射局所皮膚の組織抽出液を Zone Starch Electrophoresis によつて分画し，この各分画を生理食塩水によつて溶出し，蛋白量を Folin の phenol 試薬によつて比色定量，一部は Boyden 法によつて各分画の抗体価を測定すると，皮内へ抗原を注射した局所皮膚組織中には，γ-globulin にのみならず，β-globulin にも抗体のピークを認めたが，静脈内に抗原を注射した家兎の皮膚組織では，抗体はγ-globulin のみに認められた．

4) Cell Transfer 法によつて，Donor rabbit が Secondary response の家兎である場合，抗原注射局所の皮膚を正常家兎下組織又は腹腔内に移植すると，7日以内に Recipient 家兎に抗体産生を認め，且7日以内に抗体曲線はピークをなして下降するのを認めた．而もこの際表皮膚組織のみの移植によつては，Recipient に抗体産生を認めなかつたが，真皮層を中心とした組織の移植によつて Recipient に抗体産生を認め，真皮層に皮膚の主な抗体産生能の存在する事実を認めた．

5) 組織培養法によつては，培養液中に溶出する抗体が微量であるため，Boyden 法を以てしては，家兎皮膚の抗原注射局所皮膚を培養した場合にのみ，培養液中に抗体産生を認め，Primary response の家兎を以てしては，In Vivo の場合と異り，抗原注射局所皮膚には，抗体産生を認めることができなかつた．これは組織培養法に於ては，培養3～4日以内には，主に上皮系細胞が増殖游走してくることが観察されるので，皮膚組織培養による抗体検出方法は適当でないことが一因であろう．

以上の実験成績から皮膚自身には抗体を産生する能力をも所有し，特に経皮免疫法でこの抗体産生能力が重要な役割を果していると考えられる．